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Fertilization biology and the evolution of chitons*

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Abstract: Studies of gamete structure and fertilization biology have revealed much about the phylogeny of molluscs. Recent studies of fertilization in chitons support the view that the basal order of chitons, the Lepidopleurida, fertilize eggs, as most molluscs do, by fusing the entire sperm with the egg and transferring chromatin, mitochondria, and centrioles into the egg cytoplasm. However, current evidence suggests that all members of the order Chitonida inject only chromatin into the egg. These chitons, which include the controversial family Callochitonidae, share a series of synapomorphic characters based on their fertilization biology that makes them unique. Current evidence suggests that Callochitonidae are basal to this order and sister taxa to the remaining Chitonida, which have been divided into two suborders, the Chitonina and Acanthochitonina. New evidence indicates that Chitonina have at least two different mechanisms for penetrating the egg. One group of species has pores in the egg hull (*e.g., Chaetopleura apiculata* (Say in Conrad, 1834) and *Stenosemus albus* (Linnaeus, 1767), whereas a second group has a continuous dense layer on the surface of the egg hull that is digested by the sperm (*e.g., Rhyssoplax tulipa* (Quoy and Gaimard, 1835) and *Stenoplax conspicua* (Pilsbry, 1892)). However, the genus *Ischnochiton* Gray, 1847 appears to be polyphyletic, as several species have distinctive characters that typify other genera or families. In particular, this genus needs to be re-evaluated using modern morphological and molecular methods. All of the Chitonina have spiny-hulled eggs with narrow bases and are quite different from the second suborder, Acanthochitonina, which is characterized by large-hull cupules with wide bases. Within Acanthochitonina, some species have open-hull cupules, whereas most have closed ones. Open-cupule species lack micropores in the hull for sperm entry, whereas several closed-cupule species exhibit micropores between hull cupules. These features of the egg are accompanied by alterations in sperm structure, such as position of the mitochondria and structure of the basal body, acrosome, and flagellum. Knowledge of the gamete structure of individual species and their fertilization biology, as demonstrated here, provides a different series of characters that can help avoid mistakes that are inherent during early development of new methods, such as molecular analyses. New details of fertilization biology have made it possible to revise preliminary analyses and provide an updated phylogeny of chitons, which differs in some important respects from other recent publications.

Key words: Polyplacophora, egg, sperm, cladistics, phylogeny

We are coming to rely more and more on molecular analyses to accurately explain phylogenetic relationships (Peterson and Eernisse 2001, Okusu *et al.* 2003, Eernisse and Peterson 2004, Smith *et al.* 2004). Nevertheless, errors and inconsistencies do occur, making it useful to have a series of checks and balances in place based on a different set of characters. Prior to 1984, chiton taxonomy relied heavily on the structure of shell valves, spicules, scales, and girdle processes in order to distinguish between taxa (Smith 1960, Van Belle 1983, Kaas and Van Belle 2003), but it became apparent that some of these characters had evolved more than once by convergent evolution, which introduced some problems in classification.

Eernisse (1984) first suggested the use of egg structure and gill placement as additional characters, and Sirenko (1993) included these and produced a revised classification that divided chitons into two orders, Lepidopleurida and Chitonida, with the latter comprising two suborders, Chitonina and Acanthochitonina. A cladistic analysis by Buckland-Nicks (1995), which added characters for gamete biology at fertilization, supported this classification, differing only in positions of certain families within each suborder. In that analysis the family Callochitonidae came out as basal within Chitonina or as sister taxon to this suborder within Chitonida. *Cyanoplax* Gould, 1849 (= *Lepidochitona* Gray, 1821) did not place within Tonicellidae, as suggested by Sirenko (1993), but rather came out as a sister taxon to Acanthochitonidae. More recently Okusu *et al.* (2003) undertook a combined molecular and morphological study that provided the first detailed analysis of this kind. In general, their paper agreed with Sirenko (1993) and Buckland-Nicks (1995), but it differed in some important aspects. For example, Okusu *et al.* (2003) placed *Callochiton* Gray, 1847 (Callochitonidae) within Lepidopleurida, not in Chitonida. Furthermore, there were inconsistent results from their different molecular analyses (16sRNA, COI, and histone H3) for both *Schizochiton* Gray, 1847 and *Lepidozona* Pilsbry, 1892 (Okusu *et al.* 2003: figs. 2, 3, 4, pp. 288-291). Most

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previous classifications, including the combined morphological dataset of Okusu *et al.* (2003: fig. 8, p. 295), placed *Lepidozona* close to *Stenoplax* in Ischnochitonidae. The genus *Ischnochiton* Gray, 1847 consistently came out as being a polyphyletic taxon, with some species of the genus turning up in different families (Okusu *et al.* 2003). Furthermore, they did not find support for a monophyletic Acanthochitonina, suggesting instead that it comprised two or more paraphyletic clades.

A preliminary re-analysis of morphological data (Buckland-Nicks 2006) once again placed Callochitonidae outside the Lepidopleurida, either as sister taxon to Chitonida or basal within it, as suggested previously (Buckland-Nicks and Hodgson 2000). More recently scientists have begun looking at other potential characters for distinguishing taxa, such as mineral composition of the radula (Brooker *et al.* 2006) and the gene sequence coding for hemocyanin protein (Lieb *et al.* 2006).

This paper seeks to further elucidate the morphology of sperm and eggs at fertilization in selected key taxa and thereby provide a focus for discussion of some of these new ideas. Furthermore, previous morphological data are reanalyzed and updated with current information, providing some new insights into chiton phylogeny, which are remarkably consistent with those achieved by completely new types of analysis (Lieb *et al.* 2006). Ideally, all of these analyses should be combined to provide the most comprehensive test of phylogenetic relationships.

MATERIALS AND METHODS

Specimens

Specimens were collected from various sites around the world (see Table 1). Species of the following families are represented (terminology following Kaas *et al.* 2006 and Eernisse *et al.* 2007): Leptochitonidae (= Lepidopleuridae), Callochitonidae, Ischnochitonidae, Chitonidae, Acanthopleuridae, Lepidochitonidae, Acanthochitonidae, and Mopaliidae. Several minor families that have not been included because insufficient data are available on their reproductive biology include (terminology following Sirenko 2006): Ferreiraellidae, Nierstraszellidae, Callistoplacidae, Loricidae, Hemiarthridae, Choriplacidae, Schizochitonidae, and Cryptoplacidae.

Light and electron microscopy

Fixation for transmission electron microscopy (TEM) and scanning electron microscopy (SEM) followed methods of Buckland-Nicks and Hodgson (2000). Individual chitons were isolated in petri dishes and induced to spawn by adding sperm to each dish. Egg-laying females were removed from

Table 1. A list of chiton species collected for the study and arranged in alphabetical order (all coordinates are approximate). Where a new genus has been assigned, the old genus is shown in brackets; subgenera are not shown.

Species	Collection site/year	Latitude, longitude	Collected by
Acanthopleura granulata (Gmelin, 1791)	Trinidad 2001	10°40′N, 61°39′W	J. Buckland-Nicks (J.B.-N.)
Acanthochitona viridis (Pease, 1872)	Oahu, Hawaii 1987	21°18′N, 158°09′W	J.B.-N.
Callochiton dentatus (Spengler, 1797)	East London, S. Africa 1999	33°03′S, 28°03′E	J.B.-N. and A. Hodgson
Chaetopleura apiculata (Say in Conrad, 1834)	Florida 2004		Gulf Specimen Co., Florida
Cryptochiton stelleri (von Middendorff, 1847)	San Juan Is., Washington 1988	48°28′N, 122°54′W	J.B.-N.
Cyanoplax (Lepidochitona) dentiens (Gould, 1846)	San Juan Is., Washington 1990	48°28′N, 122°54′W	J.B.-N. and D. Eernisse
Cyanoplax (Lepidochitona) fernaldi Eernisse, 1986	San Juan Is., Washington 1990	48°28′N, 122°54′W	J.B.-N. and D. Eernisse
Deshayesiella curvata Carpenter in Pilsbry, 1892	Vostok Bay, Russia 1997	42°53′N, 132°44′E	B. Sirenko
Hanleya hanleyi W. Bean in Thorpe, 1844	Lurcher Bank, Canada 1994	43°15′N, 65°30′W	R. Mayhew
Leptochiton asellus (Gmelin, 1791)	Bergen, Norway 2006	60°20′N, 5°11′E	J.B.-N and C. Schander
Leptochiton assimilis Thiele, 1909	Vostok Bay, Russia 1997	42°53′N, 132°44′E	B. Sirenko
Leptochiton rugatus (Pilsbry, 1892)	Vostok Bay, Russia 1997	42°53′N, 132°44′E	B. Sirenko
Mopalia muscosa (Gould, 1846)	San Juan Is., Washington 1990	48°28′N, 122°54′W	J.B.-N. and D. Eernisse
Nuttallina californica (Nuttall MS, Reeve, 1847)	S. California 1993		Sea Life Supply, California
Radsia (Chiton) nigrovirescens de Blainville, 1825	Eastern Cape, S. Africa 1999	34°16′S, 18°40′E	J.B.-N.
Rhyssoplax (Chiton) tulipa Quoy and Gaimard, 1835	East London, S. Africa 1999	33°03′S, 28°03′E	J.B.-N. and A. Hodgson
Stenoplax conspicua (Pilsbry, 1892)	S. California 1993		Sea Life Supply, California
Stenosemus (Ischnochiton) albus (Linnaeus, 1767)	Bergen, Norway 2006	60°20′N, 5°11′E	J.B.-N. and C. Schander

dishes, cleaned, and replaced in clean dishes with filtered sea water. Eggs, and sometimes fertilization events, were observed with Nomarski optics. Overnight primary fixation in ice cold 2.5% glutaraldehyde in 0.1M cacodylate buffer (pH 7.4) in filtered sea water with 0.1M sucrose was followed by secondary fixation with 2% osmium tetroxide for 1 h in the same buffer. Fixed eggs were rinsed in distilled water and dehydrated in an ethanol series to 100%. Batches of eggs were divided into equal aliquots and processed separately for TEM and SEM.

For TEM, ethanol was replaced with propylene oxide and then eggs were infiltrated with either TAAB 812/Araldite or Spurrs/Epon. Samples in pure resin were left all day before baking in a 60 °C oven for two days. Thick and thin sections were cut with a diamond knife (Diatome, Switzerland). Thin sections were picked up on naked 150 mesh copper grids and stained sequentially with aqueous uranyl acetate (20 min) and lead citrate (5 min) with extensive washing with degassed distilled water between stains and after staining. Stained sections were examined in a Philips TEM 410 operated at 80 kV.

Eggs destined for SEM were aspirated into Teflon flo-thru vials (Pelco) before critical point drying, mounting on stubs using double-sided carbon tabs, and coating with gold in an SPI-Module Sputter Coater. Stubs were examined in a JEOL JSM 5300 SEM and photographed.

RESULTS

Gamete structure and fertilization in Leptochitonidae (Order Lepidopleurida)

The sperm of *Leptochiton rugatus* (Pilsbry, 1892) and *Leptochiton assimilis* Thiele, 1909 are essentially similar in structure to *Leptochiton asellus* (Gmelin, 1791) (Hodgson *et al.* 1988), having a bullet-shaped nucleus capped by an elongate acrosome cone about 3 μm in length with subacrosomal material (Figs. 1A, 1C, 2A). The proximal and distal centrioles are central and can be distinguished as separate entities although connected by flocculent material (Fig. 1B). They are surrounded by five or six large, spherical mitochondria and give rise to a central flagellum (Figs. 1A, 2A). Sperm of the lepidopleurids *Deshayesiella curvata* Carpenter in Pilsbry, 1892 and *Hanleya hanleyi* W. Bean in Thorpe, 1844 are essentially similar in terms of centrioles and mitochondria. However, in these sperm, as an extension of the nucleus, there is a short nuclear filament less than 2 μm in length, which is capped by a smaller acrosome about 1 μm long (Figs. 1D, 2B).

The eggs of *Leptochiton asellus* have a smooth jelly coat without pores (Figs. 1E, 1F, 3A), whereas those of *Deshayesiella curvata* have been shown to have large pores in the jelly coat (Pashchenko and Drozdov 1998), similar to those of *Callochiton dentatus* (Spengler, 1797) (= *Callochiton castaneus* Wood, 1815) (Fig. 3B). Observations of fertilization in *L. asellus* within ten minutes of exposure of eggs to sperm revealed that the sperm had digested the jelly coat and breached a large hole in the vitelline layer, coming to rest in the perivitelline space (Figs. 1E, 1F). This is the first time in chitons, and specifically in lepidopleurids, that the entire sperm has been shown to penetrate below the vitelline layer.

Gamete structure and fertilization in Callochitonidae (Order Chitonida)

The sperm of *Callochiton dentatus* is unlike lepidopleurid sperm because it has a nuclear filament greater than 3 μm in length, tipped by a highly reduced acrosome (Fig. 2C). Furthermore, the centrioles are fused into a slightly acentric basal body and the five mitochondria are not all spherical (Fig. 2C, 4A). This description would apply to virtually all sperm of Chitonida but none of Lepidopleurida, so far described.

The eggs of *Callochiton dentatus* are similar to those of *Deshayesiella curvata* with open pores in a thick jelly coat (Fig. 3B). Each pore coincides with a depression in the egg membrane. At fertilization the sperm nuclear filament bridges the gap between the vitelline layer and egg membrane, thus replacing the acrosomal process that characterizes Lepidopleurida and other molluscs. This is significant because in *C. dentatus*, when the acrosome digests the vitelline layer, only a tiny pore is made (Fig. 4B), which does not permit the fertilization cone to raise up and engulf the entire sperm, and it remains below the vitelline layer (Figs. 4B, 4D). Current evidence suggests that sperm mitochondria and centrioles are left on the surface of the egg.

Gamete structure and fertilization in Chitonina (Order Chitonida)

The sperm of all Chitonina have very acentric basal bodies in which the proximal centriole is positioned perpendicular and lateral (on the mitochondrial side) to the distal centriole in a fused mass (Figs. 2D, 4E, 5A, 5B). The basal body is usually in line with one side of the nucleus, with the mitochondria reduced in number to 3 or 4 on the other side. As usual, the annulus binds the distal centriole to the plasma membrane but extending from it, along the plasma membrane, mainly on the side containing the mitochondria, is a dense thickening (Fig. 2D, 4E, 5A, 5B). In addition to basal mitochondria, lateral mitochondria are present in sperm of some Chitonina, such as *Ischnochiton*, *Stenoplax*, and *Lepidozona* (Fig. 5B). Glycogen rosettes are visible in the space between mitochondria and centrioles (Figs. 2D, 4E, 5A, 5B).

All Chitonina have eggs with elaborate hulls raised into

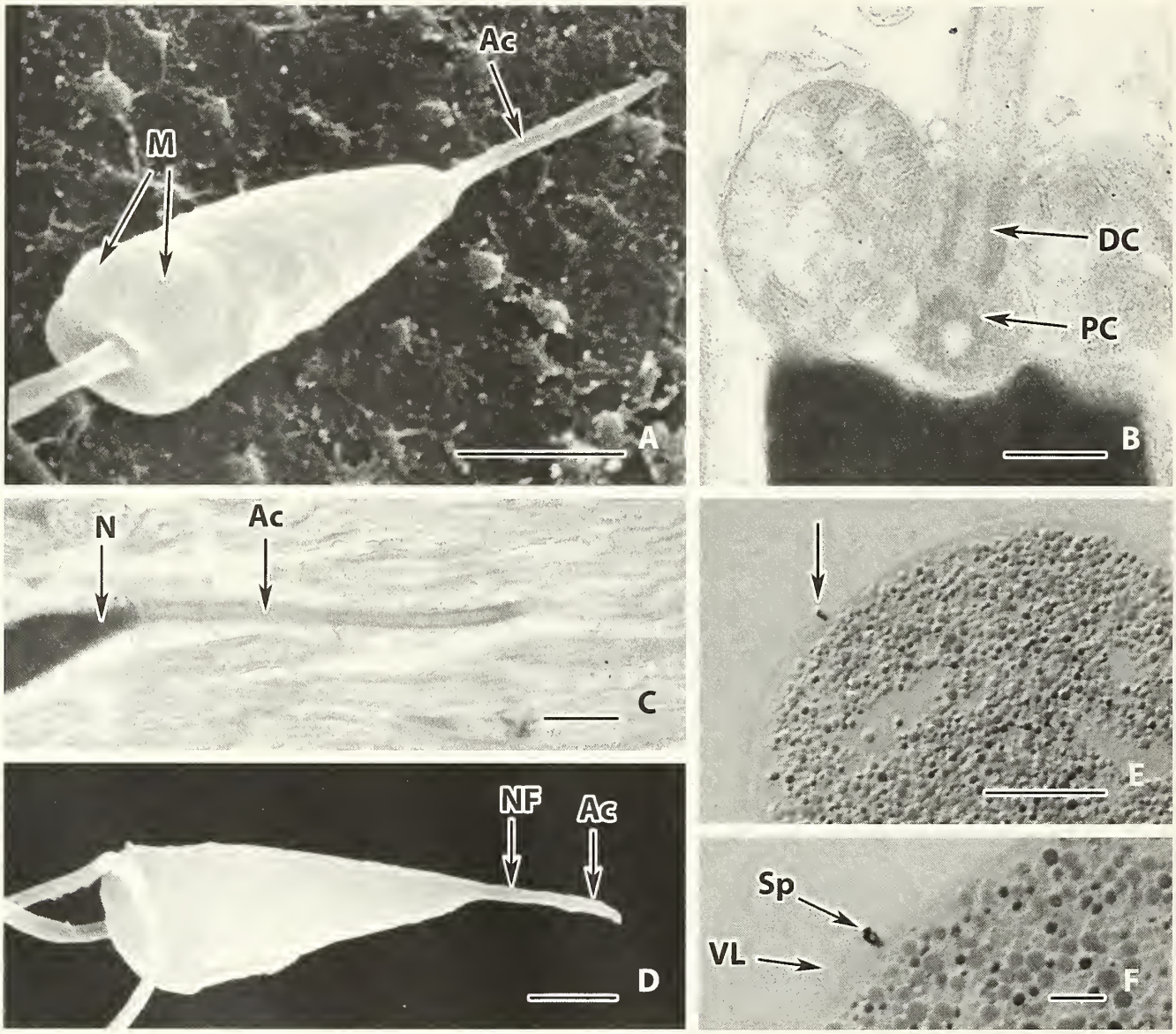

Figure 1. A, Sperm of *Leptochiton asellus* viewed with SEM. Note anterior elongate acrosome (Ac) and mitochondria (M) posterior to nucleus. Scale bar = 1 μm. B, TEM of base of sperm of *Leptochiton rugatus* showing separate proximal centriole (PC) and distal centriole (DC), adjacent to spherical mitochondrion and dense nucleus. Scale bar = 0.3 μm. C, Sperm acrosome cone (Ac) and tip of nucleus (N) of *Leptochiton assimilis*, viewed with TEM. Scale bar = 0.5 μm. D, Sperm of *Deshayesiella curvata* viewed with SEM. Note nuclear filament (NF) and acrosome (Ac). Scale bar = 1.5 μm. E, Light micrograph of 1 μm section of egg of *Leptochiton asellus* showing that entire sperm has breached vitelline layer (arrow). Scale bar = 35 μm. F, Part of E magnified to show penetrating sperm (Sp) beneath vitelline layer (VL), prior to entry into the egg. Scale bar = 7 μm.

a series of spines with narrow bases ranging in size from 5 to 30 μm (Figs. 3C, 3D, 6A-D). Two main types of fertilization have been observed. The first type involves penetration of open pores in the hull (Fig. 7B); and the second type involves sperm digestion of a thin dense layer covering the hull (Figs. 7D, 7E).

Mechanism 1: Fertilization via open pores in hull

Stenosemus albus (= *Ischnochiton albus*) and *Chaetopleura apiculata* have open pores in the egg hull, ranging in size from 1 to 4 μm, at the base of the spines. Within about thirty seconds of sperm release into a beaker of eggs, some sperm locate and penetrate these pores with their

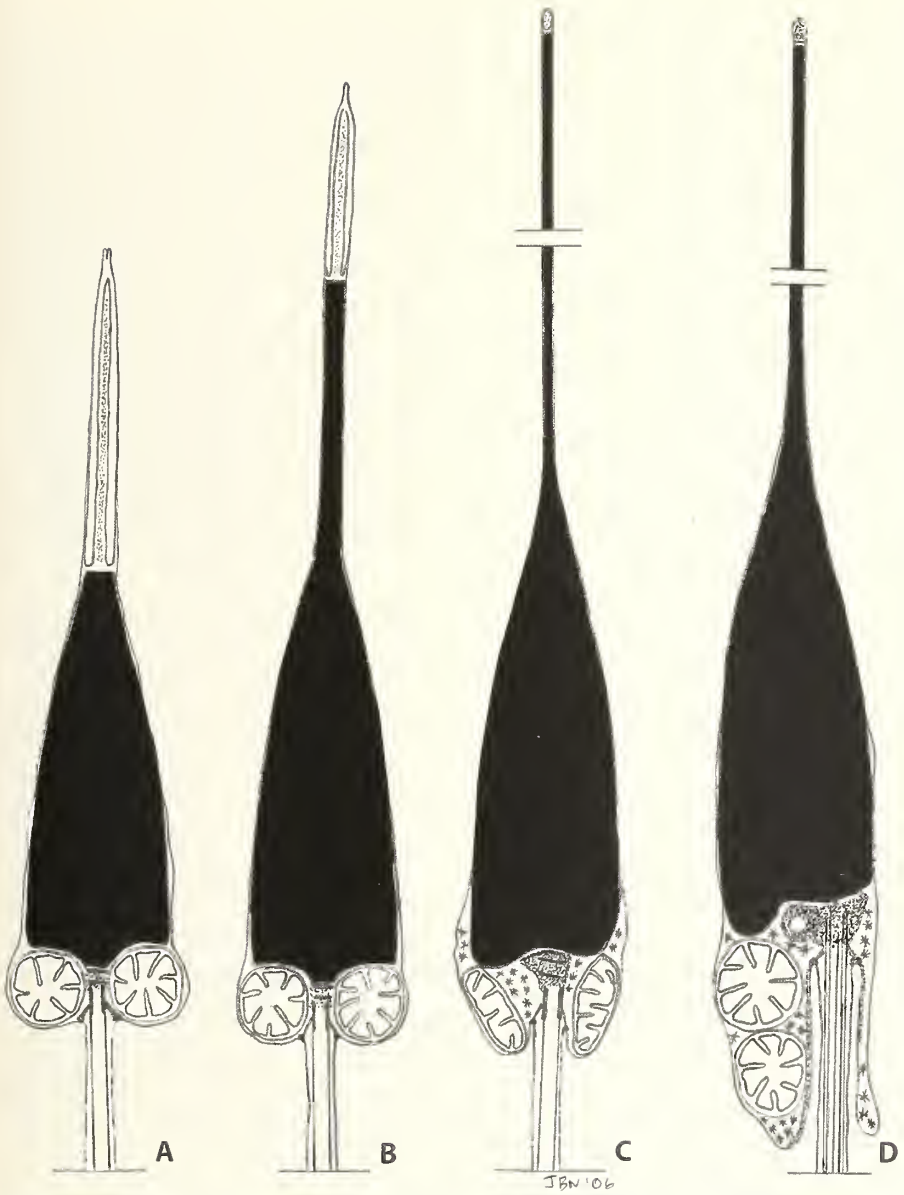

Figure 2. A, Sperm of *Leptochiton asellus*; B, sperm of *Deshayesiella curvata*; C, sperm of *Callochiton dentatus*; and D, sperm of *Chiton tuberculatus*.

anterior filament (Fig. 7B). The pores provide sperm direct access to the vitelline layer, which is digested by the acrosome before fusion occurs between sperm and egg membranes.

Hull spines of *Chaetopleura apiculata* eggs are complex with many branches off each spine (Fig. 6D). Overlapping branches between spines create complex channels that the sperm must negotiate in order to reach the base of the spines, where the pores are located. In *C. apiculata* the pores can be large enough to admit entire sperm, which sometimes gain direct access to the vitelline layer, before penetrating the egg (Buckland-Nicks and Brothers 2008).

In *Stenosemus albus* the hull comprises long spines with recurved tips (Fig. 3C). Pores are arrayed alongside the junctions of hexagonal bases, around the perimeter of each base (Fig. 7B).

Mechanism 2: Fertilization via a dense layer on hull

Rhyssoplax tulipa (= *Chiton tulipa* Quoy and Gaimard, 1835) and *Stenoplax conspicua* both have a continuous dense layer overlying the hull. This dense layer is invariably digested by the acrosome reaction on contact with the hull (Figs. 7D-E) and has been observed in numerous thick sections as well as some thin sections of both species. *Radsia nigrovirescens* (= *Chiton nigrovirescens* de Blainville, 1825) is unusual among other Chitoninae examined in having pores in the hull (Fig. 7A) although it is not known if these are used by the sperm at fertilization. Typically, brooding chitons have reduced spines or cupules, but specimens of *R. nigrovirescens* have long, simple spines with hooked tips (Fig. 3D), more like those of *Stenosemus albus*, than those of other Chitoninae.

Acanthopleura granulata (Gmelin, 1791) has unusual polymorphic spines on the egg (Fig. 6C). Some spines are short and bifurcating at the tip whereas others are intermediate or longer in length and have a scaly appearance (Fig. 6C). This egg does not appear to have pores in the hull and the sperm penetrate directly, in the same way as they do in *Stenoplax conspicua* and *Rhyssoplax tulipa*.

Gamete structure and fertilization in Acanthochitonina (Order Chitonida)

Gamete structure and fertilization in Acanthochitonina is broadly similar to that in Chitonina. However, all sperm of Acanthochitonina can be distinguished by having anterior mitochondria as well as basal and lateral ones, numbering 7 or 8 in total. Below the annulus, a fibrous complex is found

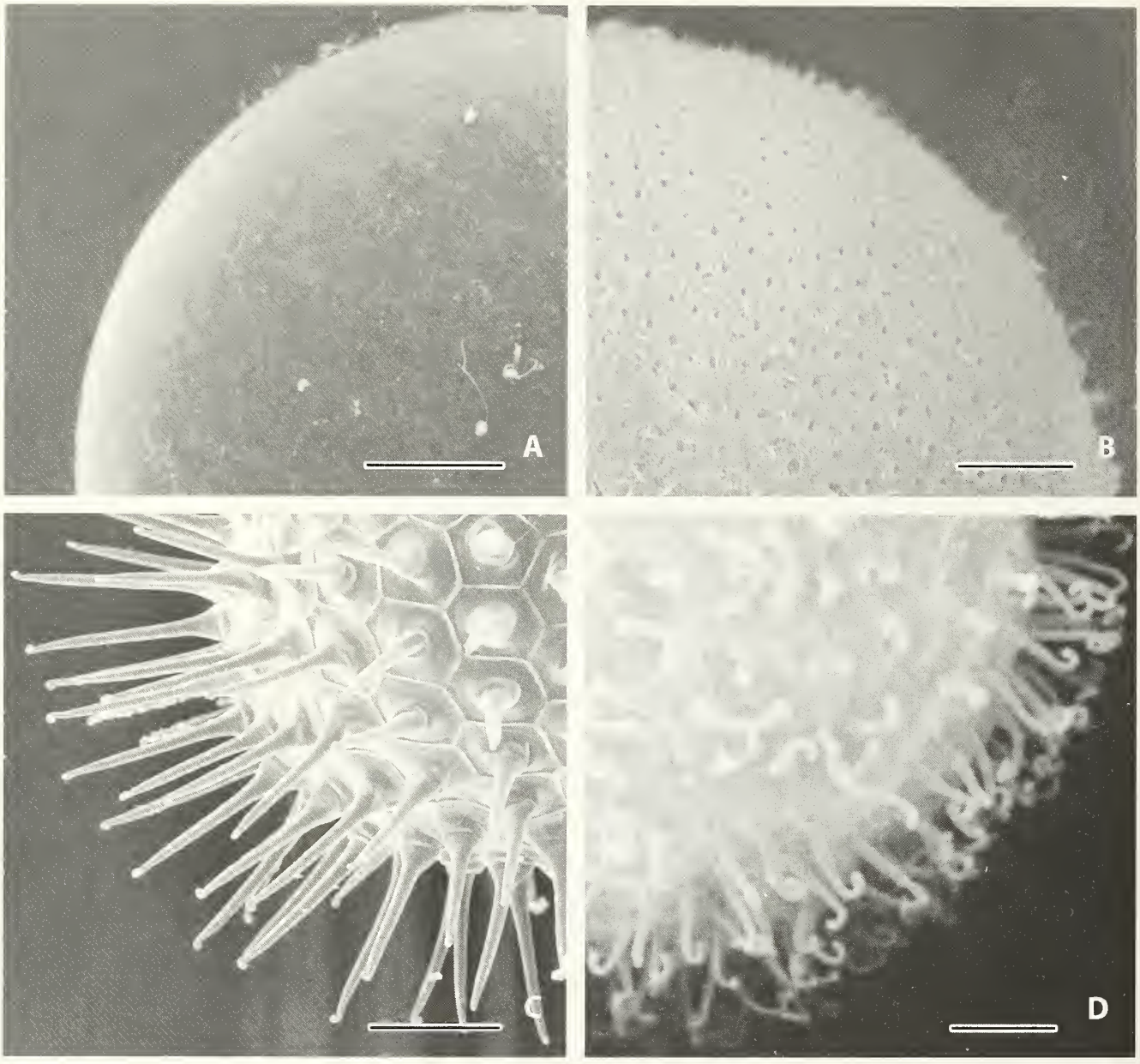

Figure 3. A, Quarter of egg of *Leptochiton asellus* viewed with SEM, showing smooth surface of coat without pores. Scale bar = 25 μm. B, Quarter of egg of *Callochiton dentatus* viewed with SEM, showing regularly spaced pores in smooth jelly coat. Scale bar = 25 μm. C, Quarter of egg of *Stenosemus albus* viewed with SEM, showing simple spines with hooked tips and hexagonal bases measuring 36 μm across. Scale bar = 50 μm. D, Quarter of egg of the brooding chiton *Radsia nigrovirescens* showing long simple spines with hooks which interlock in the pallial grooves retaining eggs. Scale bar = 50 μm.

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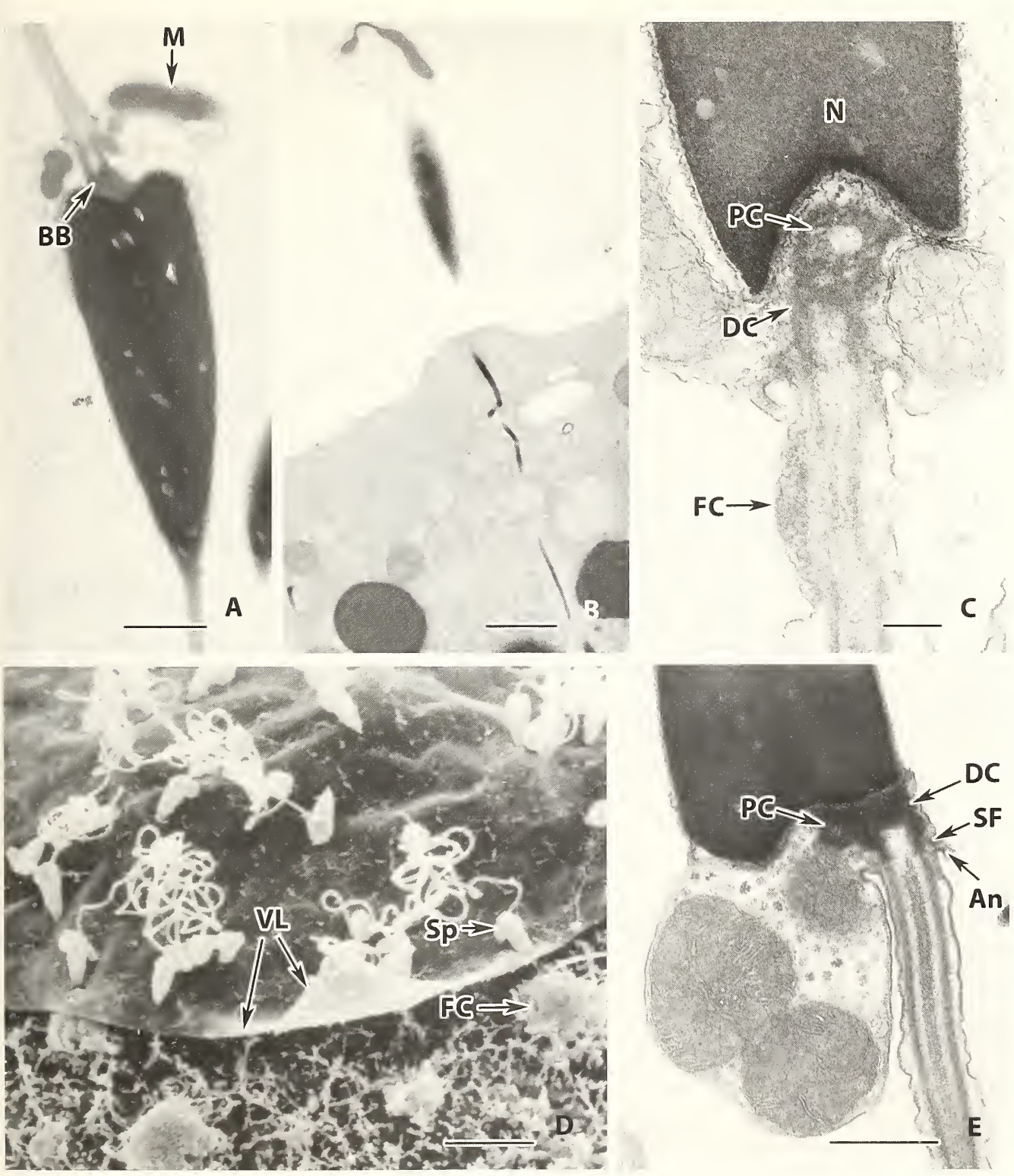
Figure 4. A, TEM of sperm of *Callochiton dentatus* showing oblong mitochondrion (M) and centriolar basal body (BB) acentric to nucleus. Scale bar = 1 μm. B, TEM of sperm penetrating egg of *C. dentatus*, showing long thread of chromatin in egg cortex. Pore in vitelline layer is too small to admit sperm organelles which, like the elongate mitochondrion, appear to be abandoned on the surface in a bag of membrane. Scale bar = 1 μm. C, TEM of sperm of *Mopalia muscosa* showing proximal centriole (PC) fused to apex of distal centriole (DC) in fossa of nucleus (N). Flagellum is reinforced by a fibrous complex (FC), which characterizes the suborder Acanthochitonina. Scale bar = 0.2 μm. D, SEM of polyspermic fertilization of *Callochiton dentatus* egg, showing multiple sperm (Sp) penetrating vitelline layer (VL)



and the induction of several fertilization cones (FC). Note: jelly layer has been completely dissolved. Scale bar = 5 μm. E, TEM of sperm of *Chaetopleura apiculata*, showing basal body comprised of proximal centriole (PC) fused laterally to distal centriole (DC), which produces an acentric flagellum. Thickening of membrane is visible extending posteriorly from annulus (An) on both sides. Mitochondria with glycogen granules are arranged in posterior extension of mid-piece. Scale bar = 0.5 μm.

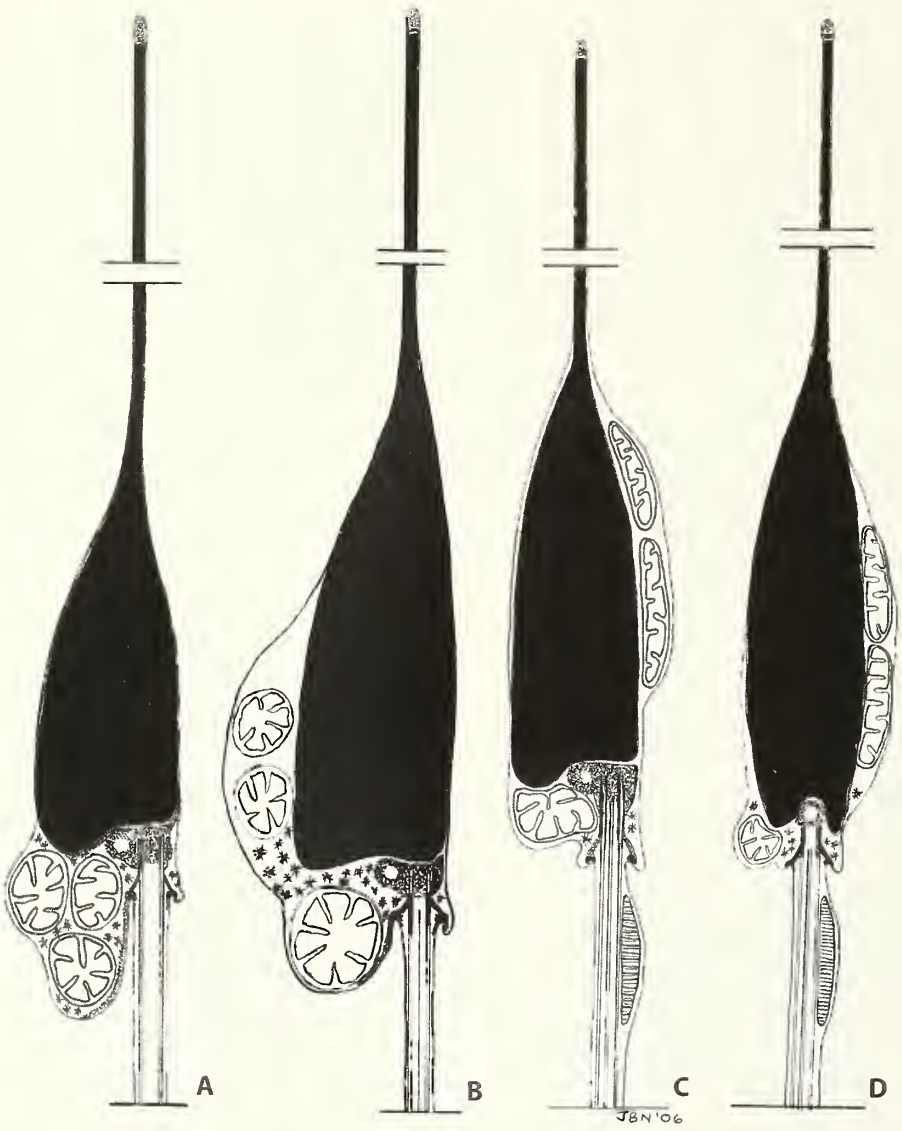


Figure 5. A, Sperm of *Chaetopleura apiculata*; B, sperm of *Stenoplax conspicua*; C, Sperm of *Acanthochitona viridis*; and D, sperm of *Mopalia muscosa*.

on the side opposite to the mitochondria (Figs. 4C, 5C-D). More specific differences in the arrangement of centrioles in the basal body and in the structure of the acrosome may distinguish among two or more groups in this suborder. For example, in *Cyanoplax* and *Acanthochitona* the proximal centriole fuses laterally with the distal centriole towards the axis of the sperm, as was found in Chitonina (Fig. 5C). However, in the genera *Tonicella* Carpenter, 1873, *Cryptochiton* Middendorff, 1847, and *Mopalia* Gray, 1847, the proximal centriole fuses to the anterior of the distal centriole and is embedded in a small nuclear fossa (Figs. 4C, 5D).

Acanthochitonina species also are united by having elaborate egg hulls raised into large cupules with wide bases ranging in size from 50 to 90 μm (Figs. 8A-D). Two main types of fertilization have been observed which are defined also by cupule morphology. The first involves closed-hull cupules with small pores in the intercupule area that give sperm direct access to the vitelline layer (Fig. 7F). The second type involves open-hull cupules with sperm swimming inside and penetrating both hull and vitelline layer without access to any pores (Fig. 7G). In both cases, the sperm inject the chromatin into the egg through the narrow nuclear filament. Other sperm organelles, including mitochondria and centrioles, appear to be excluded and left behind on the egg surface in a bag of sperm membrane (Fig. 7C).

Some species with closed cupules are brooders and in some of these, such as *Cyanoplax fernaldi* (= *Lepidochitona fernaldi* Eernisse, 1986) (Fig. 8C), the cupules are reduced but maintain the same form as non-brooding species, such as *Cyanoplax dentiens* (= *Lepidochitona dentiens* (Gould, 1846)) (Fig. 8C). Among open-cupule species some have a more complex, folded-cupule structure with protruding elements inside each cupule (Fig. 7G) (*e.g.*, the genera *Tonicella*, *Cryptochiton*, and *Mopalia* of those studied here). However, *Nuttallina californica* (Nuttall MS, Reeve, 1847) and related species do not have these protruding structures (Fig. 8D).

Cladistic analysis

Sperm and egg characters for all taxa are summarized (Tables 2-3). The data matrix (Table 2) was run through branch and bound analysis in PAUP 4.0. The Bootstrap consensus tree (for 500 replicates) resulting from this analysis is shown with confidence values written above the line for each node (Fig. 9). Lepidopleurida came out as paraphyletic in this consensus tree, largely because characters for egg and sperm shared by *Deshayesiella curvata* and *Hanleya hanleyi* were coded as apomorphic (Table 4). There was 100% support for a monophyletic Chitonida, which includes Callochitonidae as the sister taxon to Chitonina plus Acanthochi-

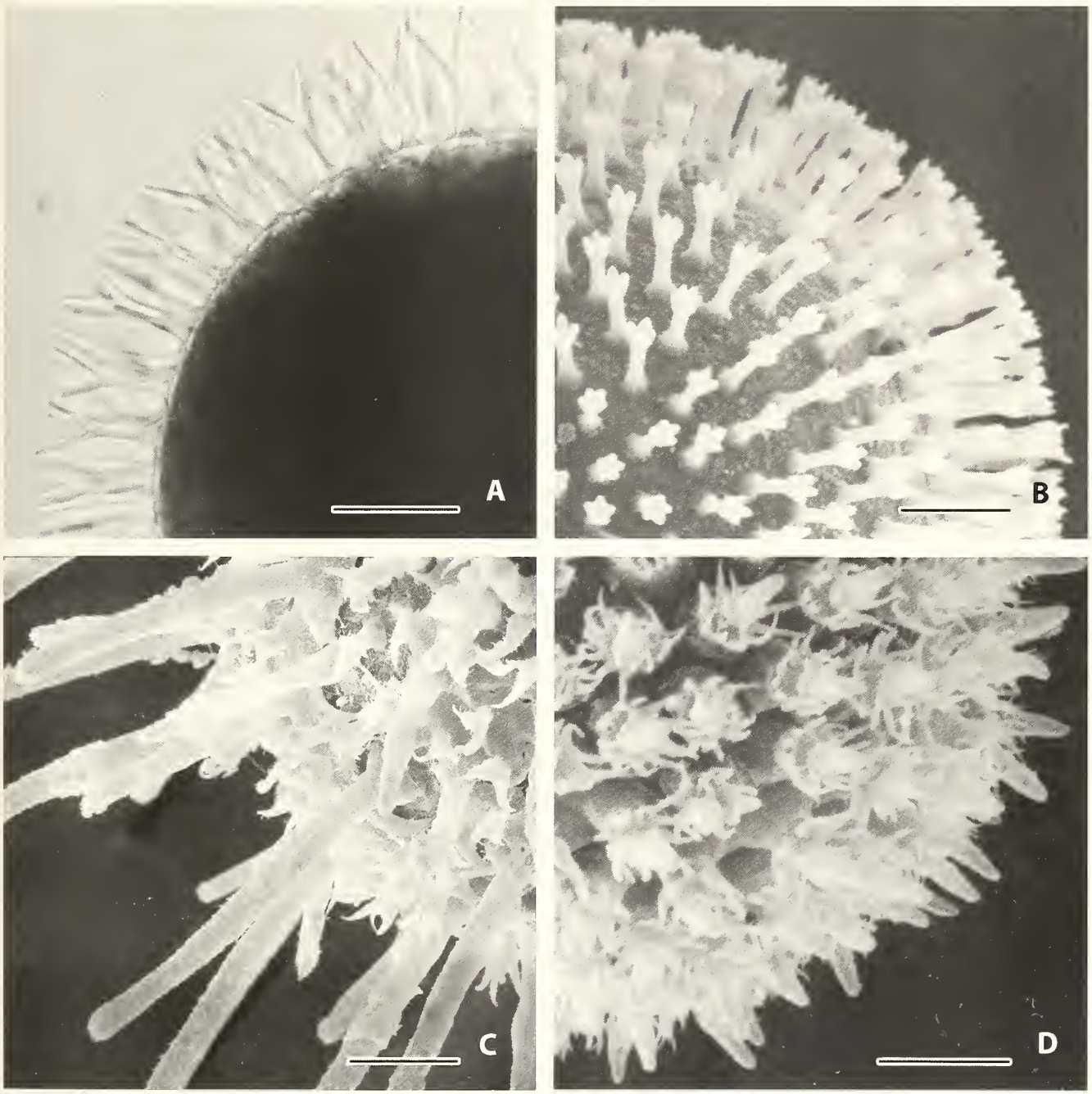


Figure 6. A, Quarter of egg of *Stenoplax conspicua* viewed with DIC optics, showing spines with bifurcating tips, which characterize this and other genera, including *Lepidozona*. Scale bar = 25 μm. B, Quarter of egg of *Rhyssoplax tulipa* viewed with SEM, showing spines with petalloid tips, typical of this genus and of some species of *Ischnochiton*. Scale bar = 20 μm. C, Quarter of egg of *Acanthopleura granulata* viewed with SEM, showing unique polymorphic spines. Some spines are elongate with a scale-like outer layer; others are intermediate in length and a third type is short and bifurcates at the tip. Scale bar = 20 μm. D, Quarter of egg of *Chaetopleura apiculata* viewed with SEM, showing complex branching spines that may be unique to this genus. Scale bar = 30 μm.

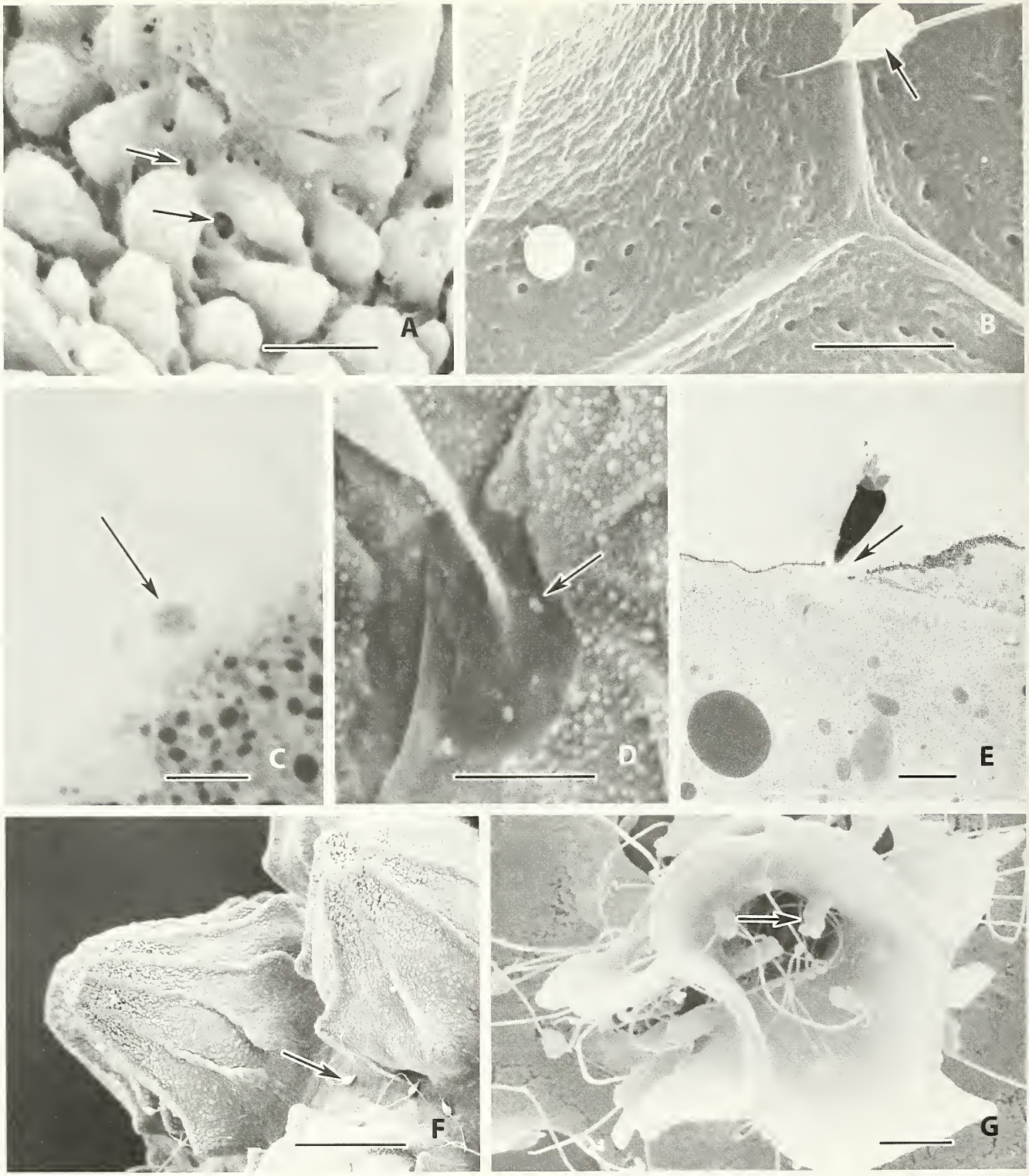


Figure 7. A, SEM detail at base of spine on egg of *Radsia nigrovirescens* showing pores in hull (arrows). Scale bar = 3 μm. B, SEM of egg of *Stenosemus albus* showing pores at perimeter of hexagonal bases of spines, which allow sperm (arrow) direct access to vitelline layer. Scale bar = 1 μm. C, LM of 1 μm section of *Mopalia muscosa* egg fertilized two hours before with dilute sperm suspension. Serial sections revealed only one membrane bag containing eight or nine particles which correspond to the roughly eight mitochondria and centrioles of this sperm (Buckland-Nicks and Brothers, unpubl. micrograph). Scale bar = 1 μm. D, SEM of sperm penetrating egg of *Stenoplax conspicua* showing

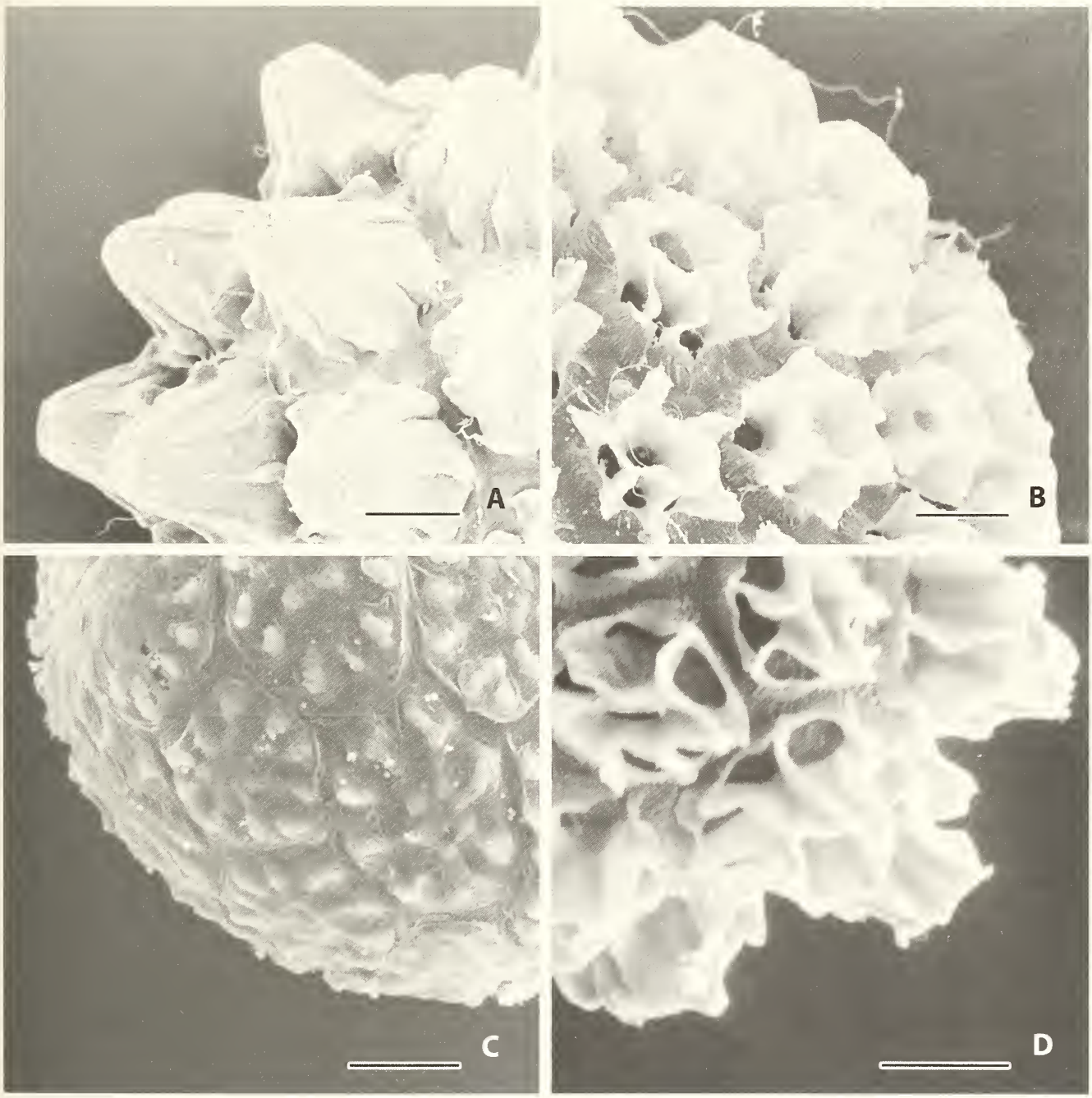


Figure 8. A, Quarter of egg of *Cyanoplax dentiens* viewed with SEM, showing closed hull cupules typical of this genus, as well as *Acanthochitona*. Scale bar = 25 μm. B, Quarter of egg of *Mopalia muscosa* viewed with SEM, showing open cupules typical of this clade. Scale bar = 25 μm. C, Quarter of egg of brooding chiton *Cyanoplax fernaldi* viewed with SEM, showing reduced hull cupules with typical hexagonal bases. Micrograph courtesy of D. Eernisse (see also Eernisse 1984). Scale bar = 30 μm. D, Quarter of egg of *Nuttallina californica* viewed with SEM, showing the type of open-hull cupules that lack internal protrusions. Scale bar = 20 μm.

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dissolution of outer dense layer around nuclear filament (arrow). Scale bar = 1 μm. E, TEM of sperm penetrating egg of *Rhyssoplax tulipa* showing dissolution of outer dense layer around nuclear filament (arrow). Scale bar = 2 μm. F, Fertilized egg of *Cyanoplax dentiens* showing penetrating sperm (arrow) between closed cupules. Scale bar = 10 μm. G, Fertilized egg of *Mopalia muscosa* showing protrusions (arrow) from inside wall of hull cupules that are absent from cupules of *Nuttallina californica*. Scale bar = 5 μm.

Table 2. Characters used in cladistic analysis of Polyplacophora and Aplacophora (outgroups) with character state codes in boldface font (see Table 3 for data matrix).

SPERM AND EGG DATA

(1) Acrosome: **0:** Acrosome forms from Golgi body in posterior of spermatid; **1:** Acrosome forms by aggregation of small proacrosome vesicles at filament tip.
(2) Acrosome structure: **0:** Cone with subacrosomal granule (SAG), interstitial granule (IG), and subacrosomal plate (SAP); **1:** Cone with SAG and SAP; **2:** Vesicle with SAP.
(3) Mitochondria position: **0:** Sheath around flagellum; **1:** Ring around centrioles; **2:** Ring around offset basal body; **3:** Basal mitochondria in collar; **4:** Lateral mitochondria; **5:** Lateral and anterior mitochondria.
(4) Mitochondria number: **0:** 1-2; **1:** 3-4; **2:** 5-6; **3:** 7-9.
(5) Mitochondrial shape: **0:** Fused spiral; **1:** All spherical; **2:** Not all spherical.
(6) Nuclear filament: **0:** Absent; **1:** 1-2 μm; **2:** ≥3 μm.
(7) Flagellum reinforcement: **0:** Absent; **1:** Spiral ribbon; **2:** Thickened membrane; **3:** Unilateral fibrous body.
(8) Chromatin pattern: **0:** Granular; **1:** Thick short fibers; **2:** Fine then coarse fibers; **3:** Fine long fibers.
(9) Centrioles: **0:** Basal body in deep nuclear fossa; **1:** Separate centrioles; **2:** Proximal centriole (PC) fused lateral to distal centriole (DC) and offset; **3:** PC fused anterior to DC in nuclear fossa.
(10) Hull projections: **0:** Absent; **1:** Spines with bases 5-30 μm; **2:** Cupules with bases 50-90 μm.
(11) Hull cupules: **0:** Absent; **1:** Closed; **2:** Open.
(12) Jelly coat: **0:** Absent; **1:** Smooth without pores; **2:** Smooth with large pores >5 μm.
(13) Hull structure: **0:** Jelly coat; **1:** Jelly coat with macropores; **2:** Hull with micropores in spines; **3:** Hull with dense layer; **4:** Hull with micropores between cupules; **5:** Cupules without pores.
(14) Fertilization site: **0:** Internal **1:** External: anywhere on egg; **2:** Sperm enters specific site.
(15) Site-specific sperm entry: **0:** Absent; **1:** Macropores >5 m; **2:** Between hull projections; **3:** Inside hull projections.
(16) Fertilization cone: **0:** Engulfs sperm and organelles; **1:** Engulfs only chromatin.

OTHER MORPHOLOGICAL DATA

(17) Gill position: **0**: Reduced; **1:** Adanal; **2:** Abanal.
(18) Gill type: **0:** Absent; **1:** Merobranchial; **2:** Holobranchial.
(19) Insertion plate: **0:** Absent; **1:** Present; **2:** Slitted; **3:** Pectinated.
(20) Body covering: **0:** Spinous; **1:** 8 shell valves.
(21) Foot: **0:** Absent; **1:** Present.
(22) Shell valves: **0:** Absent; **1:** Modern valve shape; **2:** Terminal valves with fissures.

tonina. There was also strong support (96%) for monophyly of the rest of Chitonida (= Chitonina + Acanthochitonina). However, there was weaker support (65%) for a monophyletic Chitonina, even though a number of apomorphic characters are shared by this grouping (Table 4). Also, there was similar support (62%) for a monophyletic grouping of Acanthochitonina, members of which share a different set of apomorphic characters (Table 4). However, there was strong support (97%) for a monophyletic Mopaliidae, which included *Mopalia*, *Cryptochiton*, and *Tonicella*. The apomorphy hypotheses for each of 8 selected internal nodes are listed in Table 4.

DISCUSSION

Mechanisms of fertilization

Lepidopleurida: Leptochitonidae

Confirmation of the extrusion of an acrosomal process (Buckland-Nicks 2006) and breaching of the vitelline layer by the sperm acrosome indicates that *Leptochiton asellus* penetrates the egg in a manner similar to that of most other Metazoa, including scaphopods (Dufresne-Dubé *et al.* 1983) and sea urchins (Longo 1987) although this remains to be confirmed. Other Lepidopleurida, such as *Deshayesiella curvata* and *Hanleya hanleyi*, are likely to do the same as extrusion of an acrosomal process has been observed in *D. curvata* (Buckland-Nicks, unpubl. data), and sperm of *H. hanleyi* are constructed in the same way. However, this will require confirmation by direct observation of sperm organelles following fertilization. The current analysis showed Lepidopleurida to be paraphyletic in agreement with Eernisse *et al.* (2006). However, this result depends largely on coding changes in sperm and egg structure in *Deshayesiella* and *Hanleya* as derived, rather than evolving by convergence. Recent molecular analyses (Eernisse, unpubl. data) have shown that these genera form part of a monophyletic Leptochitonidae. A combined molecular and morphological analysis would produce the most robust test of these phylogenetic relationships but for the moment we will continue to regard Lepidopleurida as the basal monophyletic order, as in Sirenko (2006).

Callochiton dentatus, although retaining the plesiomor-

Table 3. Data matrix showing characters and their character states for the species listed. A, Acrosome; As, Acrosome structure; Mp, Mitochondria position; M#, Mitochondria number; M, Mitochondria shape; Nf, Nuclear filament; Fr, Flagellum reinforcement; Ch, Chromatin pattern; Ce, Centrioles; Hp, Hull projections; Hc, Hull cupules; Jc, Jelly coat; Hs, Hull structure; Fs, Fertilization site; Se, Site specific sperm entry; Fc, Fertilization cone; Gp, Gill position; Gt, Gill type; Ip, Insertion plates; Bc, Body covering; F, Foot; Sv, Shell valves; ?, unknown state; *, outgroups.

Character type →	Ac	As	Mp	M#	M	Nf	Fr	Ch	Ce	Hp	Hc	Jc	Hs	Fs	Se	Fc	Gp	Gt	Ip	Bc	F	Sv
↓Genus/species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
*Epimenia australis**	0	0	0	0	0	0	1	?	0	0	0	1	01	0	0	?	0	0	0	0	0	0
*Chaetoderma argenteum**	0	2	1	2	1	0	0	0	1	0	0	1	0	1	0	?	0	0	0	0	0	0
Leptochiton asellus	0	1	1	2	1	0	0	1	1	0	0	1	0	1	0	0	1	1	0	1	1	1
Deshayesiella curvata	0	1	1	2	1	1	0	1	1	0	0	1	1	2	1	0	1	1	0	1	1	1
Hanleya hanleyi	0	1	1	2	1	1	0	1	1	0	0	1	1	2	1	0	1	1	1	1	1	1
Callochiton dentatus	1	2	2	2	2	2	0	2	2	0	0	1	1	2	1	1	1	1	2	1	1	1
Chaetopleura apiculata	1	2	3	1	2	2	2	2	2	1	0	0	2	2	2	1	1	2	2	1	1	2
Stenosemus albus	1	2	3	1	2	2	2	2	2	1	0	0	2	2	2	1	1	2	2	1	1	2
Acanthopleura granulata	1	2	3	1	2	2	2	2	2	1	0	0	2	2	2	1	1	2	3	1	1	2
Rhyssoplax tulipa	1	2	3	1	2	2	2	2	2	1	0	0	2	2	2	1	1	2	3	1	1	2
Ischnochiton hakodadensis	1	2	4	1	2	2	2	2	2	1	0	0	2	2	2	1	1	2	2	1	1	2
Stenoplax conspicua	1	2	4	1	2	2	2	2	2	1	0	0	2	2	2	1	1	2	2	1	1	2
Lepidozona retiporosa	1	2	4	1	2	2	2	2	2	1	0	0	2	2	2	1	1	2	2	1	1	2
Cyanoplax dentiens	1	2	5	3	2	2	3	3	2	2	1	0	3	2	2	1	2	1	2	1	1	2
Acanthochitona viridis	1	2	5	3	2	2	3	3	2	2	1	0	3	2	2	1	2	1	2	1	1	2
Tonicella lineata	1	2	5	3	2	2	3	3	3	2	2	0	4	2	3	1	2	2	2	1	1	2
Cryptochiton stelleri	1	2	5	3	2	2	3	3	3	2	2	0	4	2	3	1	2	2	2	1	1	2
Mopalia muscosa	1	2	5	3	2	2	3	3	3	2	2	0	4	2	3	1	2	2	2	1	1	2

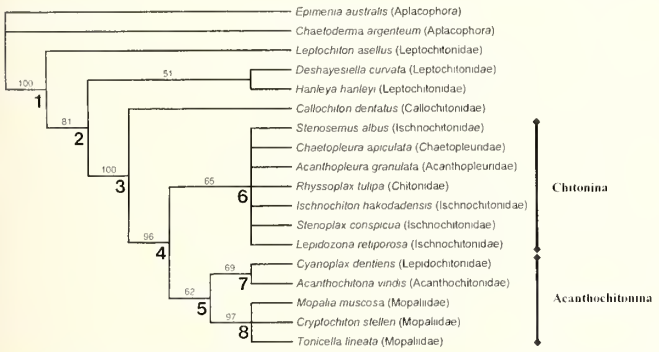

Figure 9. Consensus tree from branch and bound analysis in PAUP 4.0 of the data matrix in Table 2. Numbers above nodes represent the bootstrap values for 500 replicates. Numbers below refer to internal nodes. Apomorphy hypotheses describing these nodes are listed in Table 4.

Table 4. Apomorphy hypotheses for selected internal nodes of bootstrap consensus tree shown in Fig. 9. Unless otherwise stated in brackets, character changes are from 0 →1.

Internal node	Character changes
Node 1: Polyplacophora	17, 18, 20, 21, 22
Node 2: *Deshayesiella* + *Hanleya*	6
Node 3: Chitonida	1, 2 (1-2), 3 (1-2), 5 (1-2), 6 (1-2), 8 (1-2), 9(1-2), 16, 19 (1-2)
Node 4: Chitonina + Acanthochitonina	3 (2-3), 4 (2-1), 10, 12 (1-0), 15 (1-2), 18 (1-2), 22 (1-2)
Node 5: Acanthochitonina	3 (4-5), 4 (1-3), 7 (2-3), 8 (2-3), 10 (1-2), 11, 13 (2-3), 17 (1-2)
Node 6: Chitonina	7 (0-2), 13 (1-2)
Node 7: *Cyanoplax* + *Acanthochitona*	11, 13 (3-4), 18 (2-1)
Node 8: *Mopalia* + *Cryptochiton* + *Tonicella*	9 (2-3), 11 (1-2), 13 (4-5), 15 (2-3)

phic egg type first discovered in *Deshayesiella curvata* (Pashchenko and Drozdov 1998), has evolved several apomorphic features in the sperm, which dictate a novel mechanism of fertilization, and one that characterizes the order Chitonida (Buckland-Nicks and Hodgson 2000, Buckland-Nicks 2006). In this mechanism a permanent nuclear filament has replaced the extrusion of an acrosomal process; after the tiny acrosome digests a small hole in the vitelline layer, the nuclear filament fuses with an egg microvillus and delivers the chromatin into the egg. Furthermore, current evidence suggests that other sperm organelles, including mitochondria and centrioles, do not pass through this minute tube and are left behind on the surface (Buckland-Nicks and Hodgson 2000, Buckland-Nicks 2006, Buckland-Nicks and Brothers 2008). These characteristics place Callochitonidae firmly within Chitonida and outside the Lepidopleurida

(Fig. 9). This result is different than that obtained by one molecular analysis (Okusu *et al.* 2003) but agrees with previous results based on morphological data (Buckland-Nicks 1995, 2006, Sirenko 1993, 2006) or DNA sequences encoding for hemocyanin protein (Lieb *et al.* 2006).

Chitonida: Chitonina

The suborder Chitonina is unified by synapomorphic gamete characters (Table 4). In the sperm, these include the very acentric basal bodies with proximal centrioles positioned perpendicular and lateral to the distal centriole in a fused mass towards the mitochondrial side (see also Hodgson *et al.* 1988) and a dense thickening that extends from the annulus along the plasma membrane adjacent to the mitochondria (Buckland-Nicks 2006). In the egg, synapomorphic characters include hull spines with narrow bases (Sirenko 1993, 2006, Buckland-Nicks 2006). New data on fertilization in *Chaetopleura apiculata* (Buckland-Nicks and Brothers 2008) and *Stenosemus albus* indicate that they and their close relatives are more basal among Chitonina, a result which was found also by Okusu *et al.* (2003) in their combined molecular analysis (p. 293, fig. 6) and combined morphological analysis (p. 295, fig. 8). However, the consensus tree in the present study found weak support (65%) for a monophyletic Chitonina and was unable to distinguish between the families in this suborder. A combined molecular and morphological analysis would likely provide a more robust test with better resolution.

This study supports the finding of Okusu *et al.* (2003) that the genus *Ischnochiton* is polyphyletic. Okusu *et al.* (2003) showed that *Ischnochiton rissoi* (Payraudeau, 1826) is closely related to Chaetopleurinae whereas other *Ischnochiton* species came out within Ischnochitoninae. Spine form in general seems to be a good indicator of relationship among genera such as *Stenoplax* and *Lepidozona* (bifurcating spines) as well as *Chiton* Linnaeus, 1758 and *Rhyssoplax* Thiele, 1893 (petalloid spines), whereas the mosaic of spine form found among different species of *Ischnochiton* suggests polyphyly (see diagrams by Sirenko 1993 and 2006). The present study supports reclassification of *Ischnochiton albus* (Linnaeus, 1767) as *Stenosemus albus* (see Sirenko 2006). Only further studies on the reproductive biology and molecular biology of other species of *Ischnochiton* will resolve any further inconsistencies in classification.

Radsia nigrovirescens is an anomaly among Chitoninae as it has pores in the hull and an unusual spine form, more like that of *Stenosemus albus*. Furthermore, it is a brooder and in most brooders the spines are reduced or absent (Eernisse 1984, Buckland-Nicks and Eernisse 1992). Fertilization biology has not yet been studied and it remains unknown if the sperm penetrate these pores, as occurs in *Stenosemus* and *Chaetopleura*, or digest the egg hull to make an open pathway, as in other Chitoninae (Buckland-Nicks 2006).

Acanthopleura granulata is unique among chitons examined to date, as it has evolved polymorphic spines, which include long and intermediate scaly ones, as well as short bifurcating ones. Previously, eggs of *A. granulata* were illustrated with straight spines ending in points (Sirenko 1993, 2006), which is clearly a simplification. A recent analysis of a related species, *Acanthopleura echinatus* Barnes, 1824 (Gaymer *et al.* 2004), revealed spines with petalloid tips, like some other genera of Chitoninae, including *Chiton* and *Rhyssoplax*. In keeping with this, the hull does not have pores like *Chaetopleura* and *Stenosemus*, suggesting that the mechanism of fertilization is more like that in genera of Chitoninae, such as *Chiton*, *Rhyssoplax*, or of Ischnochitoninae, such as *Stenoplax* and *Lepidozona*. However, a recent molecular analysis (Eernisse, unpubl. data) indicates that Acanthopleurinae + Toniciinae is separate from Chitoninae.

Chitonida: Acanthochitonina

Members of the suborder Acanthochitonina share synapomorphies for sperm, with a fibrous complex on the flagellum and anterior mitochondria, and for eggs, with broad based cupules. Also this suborder is characterized by abanal gills (Sirenko 1993, 2006). However, two groups emerge within this suborder, one united by closed cupules, laterally fused centrioles and fertilization between cupules (*e.g.*, *Cyanoplax* Pilsbry, 1892 and *Acanthochitona* Gray, 1821), and the second united by open cupules, anteriorly fused centrioles, and fertilization inside cupules (*Mopalia*, *Tonicella*, *Cryptochiton*) (see Table 4). *Cyanoplax cinerea* (= *Lepidochitona cinerea* Linnaeus, 1767) may represent an exception to this, as it is reported to have open cupules (Eernisse 1984). In the present analysis, *Cyanoplax* places outside Mopaliidae, as was reported by Buckland-Nicks (1995) and Okusu *et al.* (2003: figs. 6, 8). This disagrees with Sirenko (2006), who placed this genus within Tonicellidae. Furthermore, in this analysis *Cryptochiton* comes out with *Mopalia* and *Tonicella* within Mopaliidae, a placement which agrees with Okusu *et al.* (2003: figs. 6, 8) and Eernisse (pers. comm.). However, Sirenko (2006) placed *Cryptochiton* in Acanthochitonidae. Okusu *et al.* (2003: fig. 7B) suggested that there is a possibility that Acanthochitonina species are paraphyletic based on one of two consensus trees for combined molecular data. However, based on morphology alone for the genera included here, this study finds some support (62%) for a monophyletic Acanthochitonina, as was also shown by the second consensus tree of Okusu *et al.* (fig. 7A: 2003).

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